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Mar. 25th, 2021
Bernadette Juarez
APHIS Deputy Administrator
Biotechnology Regulatory Services
4700 River Rd, Unit 98
Riverdale, MD 20737
Re: Request for Confirmation of Exemption

Dear Ms. Juarez,

Bioheuris Inc. respectfully requests confirmation from the USDA-APHIS's Biotechnology Regulatory Services (BRS) regarding the regulatory status of a soybean we intend to develop using CRISPR-Cas9 gene editing technology. The proposed soybean product contains one targeted base pair substitution (edits) in the Acetolactate Synthase (*ALS*) gene[1] and is tolerant to herbicides that otherwise kill plants through inhibition of the essential ALS protein. The request is described as follows.

A. Requestor's name and contact information
Quan Zhang,
Bioheuris Inc.
1100 Corporate Square Dr., St. Louis, MO 63132
Email: quan.zhang@bioheuris.com
B. Description of plant's genus, species
   - Order:*Fabales*
   - Family: *Fabaceae*
   - Genus: *Glycine*
   - Species: *Glycine max* (L.) Merr
C. Regulatory exemption we are claiming
   As described below, our intended final plant line has one amino acid substitution resulting from only one base pair change made to the soybean genome. We think that our product concept is eligible for exemption from regulation under the new BRS SECURE rule published in 7 CFR part 340 section[2], more specifically §340.1 (b)(2), which states that "A plant that contains a single modification of a targeted single base pair substitution" could be exempted from regulation.
D. Description of the trait
   The gene targeted for genome editing is *als1*(GLYMA_04G196100), which encodes for Acetolactate Synthase that catalyzes the synthesis of the branched-chain amino acids, and is present in the chloroplasts of all plant species. The trait introduced by gene editing is Herbicide resistance.
E. Description of reagents used for genome editing
   Biolistic transformation with preassembled ribonucleoprotein (synthetic gRNA + Cas9 protein) and a DNA molecule serving as a repair template including the single base target mutation. The commercially prepared ribonucleoprotein is pretreated with DNase I to

remove trace amounts of expression constructs. Furthermore, construct specific PCR will be conducted to confirm that there are no amplifications from the edited crop genome. To make sure there are no spurious DNA insertions from the ssDNA repair template, qPCR using primers specifically designed to amplify the repair ssDNA, combined with specific locked nucleic acid probes (IDT), will be conducted to confirm that there is only a single copy presented in the edited crop genome. Any tandem insertion or multiple insertion events will be discarded and only single copy events will be used for crop production. Following this, a Sanger sequencing of the targeted-for-editing region of als1 (illustrated in Figure 2) will be conducted on the edited crop genome to further confirm that the ssDNA is indeed used once as the repair template for homology-directed repair (HDR) following CRISPR-induced double stranded DNA break (DSB).

F. Description of the intended genetic modification

A DNA double strand break (DSB) will be made within the *als* gene using Cas9 nuclease[3,4], and DSB will be repaired through a homology-directed repair (HDR) pathway in the presence of the supplied DNA template. The DNA repair template (ssDNA) consisted of the exact same sequence as the targeted sequence near the DSB except for the specific base pair substitutions and flanked on each side by homology arms (sequences identical to the sequences immediately upstream and downstream of the genomic sequence to be edited) (Figure 1). The P178S substitution is accomplished by changing [C] in the Proline coding triplet ([CCC]) to [T] in the Serine coding triplet ([TCC]). The Cas9 PAM sequence (CCC) partially overlapped the Proline codon, and as a result the intended mutation mentioned above will disrupt the PAM sequence after HDR. This is a typical approach to prevent the genomic DNA from being re-cut by Cas9 after HDR occurred as its sequence could otherwise have the possibility to be recognized by gRNA-Cas9 RNP complex.

Figure 1. Sequence rendering shows the partial sequence of soybean genome sequence, repair DNA (blue) and gRNA (gRNA149) directing Cas9 nuclease to make the double stranded break (DSB). Targeted base pair substitution made to generate the BH-ALS-02 allele is shown, which results in one amino acid change P178S. The arrowhead marks the end of the ssDNA and is a graphical indication of the sequence direction.

G. Description of the screening tests to confirm the targeted gene editing of *als1* gene

There are three additional acetolactate synthase gene paralogs in the soybean genome which share high DNA sequence homology with *als1* (GLYMA_04G196100). A partial sequence alignment of these four genes is represented in Figure 2 to show the high sequence homology and the region targeted for gene editing. To ensure that only *als1* has been edited in our final product, all four genes will be subjected to Sanger sequencing at the corresponding position. In addition, *als1* gene specific primers (as illustrated in Figure 2) combined with locked nucleic acid probes (IDT) will be designed, and used to specifically confirm that there is only one nucleotide replacement (using ssDNA illustrated in Figure 1) at the ALS1 target site.

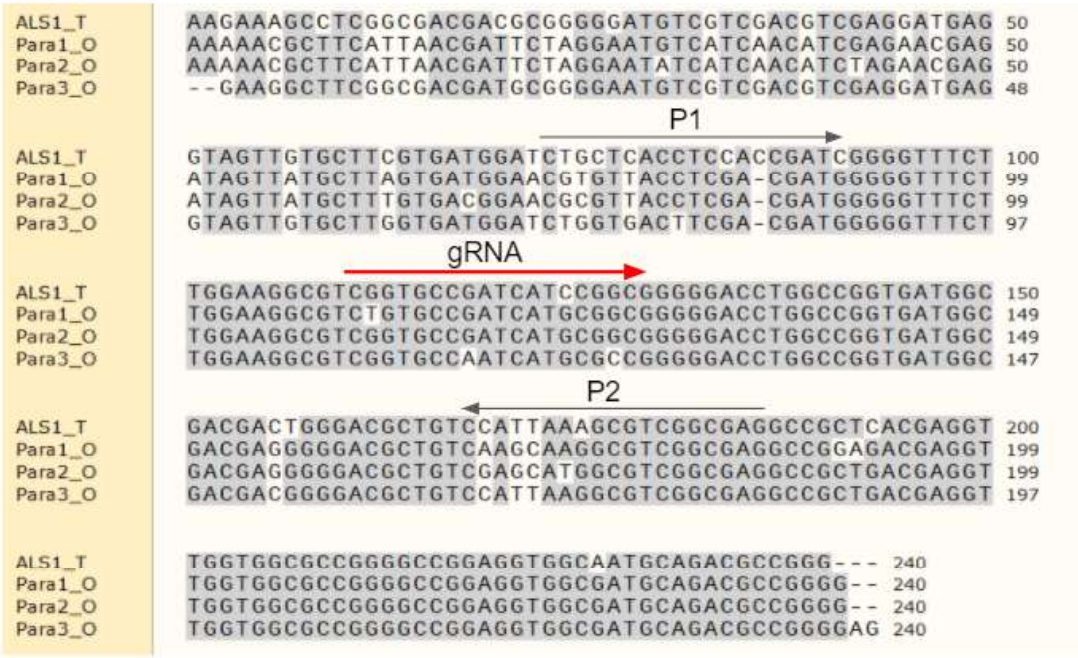

Figure 2. Partial sequence alignment of soybean ALS1 and three paralogs (potential off-targets). **ALS1_T**: ALS1 gene, GLYMA_04G196100, Target; **Para1_O**: ALS1 paralog, Glyma.13G241000, Off target; **Para2_O**: ALS1 paralog, Glyma.15G072500, Off target; **Para3_O:** ALS1 paralog, Glyma.06G169700, Off target; **gRNA:** gRNA designed specifically to guide nuclease to the ALS1_T target site for gene editing; **P1, P2:** primers for specific amplification of ALS1_T target sequence for qPCR copy number confirmation.

H. References

1. Kay L Walter, Stephen D Strachan, Nancy M Ferry, Henrik H Albert, Linda A Castle and Scott A Sebastian. Molecular and phenotypic characterization of Als1 and Als2 mutations conferring tolerance to acetolactate synthase herbicides in soybean.
2. https://www.aphis.usda.gov/brs/fedregister/BRS_2020518.pdf

3. Svitashev, Sergei, Christine Schwartz, Brian Lenderts, Joshua K. Young, and A. Mark Cigan. “Genome Editing in Maize Directed by CRISPR–Cas9 Ribonucleoprotein Complexes.” Nature Communications 7 (November 16, 2016): 13274. doi:10.1038/ncomms13274.
4. Li, Zhongsen, Zhan-Bin Liu, Aiqiu Xing, Bryan P. Moon, Jessica P. Koellhoffer, Lingxia Huang, R. Timothy Ward, Elizabeth Clifton, S. Carl Falco, and A. Mark Cigan. “Cas9-Guide RNA Directed Genome Editing in Soybean.” Plant Physiology 169, no. 2 (2015): 960–970.

Thank you for your time in reviewing this request and I look forward to your response.

Sincerely Yours,

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